# TELOMERE ELONGATION

This application claims priority from Provisional Application. No. 60/466,427, filed April 30, 2003, the content of which is incorporated herein by reference.

## TECHNICAL FIELD

The present invention relates, in general, to telomeres, and, in particular, to a method or effecting telomere elongation in mammalian cells. The invention further relates to a protein suitable for use in such a method and to nucleic acid sequences encoding same.

## BACKGROUND

Loss of telomeres limits the lifespan of human cells and thus greatly impedes the ability of mammalian tissues to be manipulated for the purposes of, for example, tissue engineering. It has been demonstrated that ectopic expression of hTERT (the catalytic protein subunit of human telomerase reverse transcriptase) in human cells can arrest telomere shortening and immortalize cells. However, sustained expression of hTERT can promote tumor growth. Thus, use in humans of cells manipulated by sustained expression of hTERT raises concerns relating to the induction of tumorigenic growth of manipulated cells. This problem could be overcome

by transient expression of hTERT, however, transient expression may not result in sufficient elongation of telomeres to provide for extended culturing.

The present invention provides a safe and efficient method of elongating telomere length of cells, e.g., cultured human cells. The method makes possible, for example, a variety of tissue engineering techniques.

## SUMMARY OF THE INVENTION

In general, the present invention relates to telomeres. More specifically, the invention relates to a method or effecting telomere elongation in mammalian cells. In addition, the invention relates to a protein suitable for use in such a method and to nucleic acid sequences encoding same.

Objects and advantages of the present invention will be clear from the description that follows.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleotide sequence of encoded hPot1-hTERT fusion protein (SEQ ID NO:1).

Figures 2A and 2B. hTERT proteins retain telomerase activity when fused to hPot1. (Fig. 1A) Lysates from HA5 cell expressing the described constructs were immunoblotted with anti-flag antibodies to detect ectopic hTERT containing proteins. Actin serves a loading control. (Fig.1B)

Lysates were assayed for *in vitro* telomerase activity. The internal standard (IS) served as a positive control for PCR amplification.

Figure 3. The hPot1-hTERT chimeric proteins elongate telomeres. Restriction enzyme-digested genomic DNA isolated from late passage HA5 cells expressing hTERT, hPot-hTERT or hPot-hTERT$_{+128}$ were hybridized with a telomeric probe to visualize telomere containing fragments. Left, molecular weight markers in kilo bases (Kb). Sample for hPot-hTERT was underloaded (*).

Figure 4. HA5 cells expressing hPot-hTERT fusion proteins immortalize. The lifespan in population doublings (pd) of HA5 cell lines infected with vectors expressing hPot-hTERT (□), hPot-hTERT$_{+128}$ (O) or controls expressing vector alone (▲), hTERT (■) or hTERT$_{+128}$ (•) is plotted against time in days.

## DETAILED DESCRIPTION OF THE INVENTION

hTERT is known to elongate telomeres when introduced into human cells (Collins et al, Oncogene 21:564-579 (2002)). However, this enzyme cannot extend telomeric DNA extensively in many human cells. The present invention provides a chimeric molecule, and a nucleic acid sequence encoding same, that can greatly elongate telomeres. The chimeric molecule comprises a protein having telomerase

catalytic activity fused to a telomere binding protein.

Advantageously, the protein having telomerase catalytic activity is the catalytic protein subunit of telomerase reverse transcriptase, for example, mammalian telomerase reverse transcriptase, e.g., human telomerase reverse transcriptase (hTERT) (GENBANK DNA accession #AH007699; GENBANK protein sequence accession #BAA74724), or functional portion or variant thereof. Examples of such portions/variants are given in USPs 5,770,422, 5,917,025, 6,093,809, 6,166,178, 6,261,836, 6,309,867, and 6,337,200.

The telomere binding protein is, advantageously, Pot1 (e.g., hPot1), or functional portion or variant thereof (see U.S. Appln. No. 20020137703), or other telomere binding or telomere associated protein such as TRF1 (Chong et al, Science 270:1663-1667 (1995)), TRF2 (Broccoli et al, Nat. Genet. 17:231-235 (1997)), PinX1 (Zhou et al, Cell 107:347-359 (2001)), Rap1 (Li et al, Cell 101:471-483 (2000)), Tin2 (Kim et al, Nat. Genet. 23:405-412 (1999)), Tankyrase (Smith et al, Science 282:1484-1487 (1998)), TANK2 (Kaminker et al, J. Biol. Chem. 276:35891-35899 (2001)) or Ku70/80 (Hsu et al, Proc. Natl. Acad. Sci. USA 96:12454-12458 (1999)), or functional portion or variant thereof (see also Armbruster et al, Mol. Cell. Biol. 23:3237-3246 (2003)). The binding protein can be present in the chimeric molecule N-terminal or C-terminal to the protein having telomerase catalytic

activity, however, N-terminal is preferred. The two components of the chimeric molecule can be linked directly or indirectly, e.g., via a spacer polypeptide or peptide or by homo- or heterodimerization via fusion to other proteins.

The invention also relates to nucleic acid sequences encoding the above-described chimeric molecule, the sequence given in Figure 1 (SEQ ID NO:1) being an example. Advantageously, the encoding sequence is present in an expression construct, typically in operable linkage with a promoter. Preferred promoters include high transcription rate promoters, such as any viral promoter (e.g., SV40) or a tetracycline-inducible promoter. (See also U. S. Application No. 10/388,588, incorporated herein by reference, and particularly disclosure therein relating to construct components).

Any of the above protein/nucleic acid sequences can be prepared chemically or recombinantly using standard techniques.

The nucleic acids of the invention can be introduced into any of a variety of cell types, for example, to provide constituent cells of required tissues. The introduction can be effected using any of a variety of approaches, which can vary with the nature of the construct employed and cell type targeted. For example, viral vectors can be used to introduce the nucleic acids of the invention into target cells (e.g., retroviral vectors, adeno-associated viral vectors, lentiviral vectors and

adenoviral vectors). Non-viral delivery methods can also be used to introduce constructs containing the nucleic acids of the invention into target cells. For example, liposome-encapsulated constructs can be used as can polymer-encapsulated constructs, receptor-mediated transfer of such encapsulated constructs, polymer-complexed constructs, constructs incorporated by electroporation, constructs incorporated by calcium phosphate precipitation, and naked constructs (Templeton et al, Gene Therapy: therapeutic mechanisms and strategies, New York (2000), Pasi, British Journal of Haematology 115:744-757 (2001)). (See also U. S. Application No. 10/388,588.)

Any of a variety of cell types can be modified to include the nucleic acids of the invention (examples of such cells include stem/progenitor cells, epithelial cells, fibroblasts, muscle cells, nervous system cells, keratinocytes, etc.). Introduction of the nucleic acids of the invention can be effected to immortalize cells, cells so immortalized being useful, for example, in tissue engineering (e.g., to produce vascular grafts - see U.S. Appln. No. 10/388,588) (Counter et al, Lancet 361:1345-1346 (2003), McKee et al, EMBO Reports, in press (2003)), bone marrow transplants (Shi et al, Nat. Biotechnol. 20:587-591 (2002)). Immortalized cells can also be used in the treatment of diseases or conditions where there is a significant cell turnover (e.g., engraftment of burns or liver disease).

As noted above, hTERT is activated in cancer cells (Collins and Mitchell, Oncogene 21:564-579 (2002), Meyerson et al, Cell 90:785-795 (1997)) and is known to be a step in tumorigenesis (Collins and Mitchell, Oncogene 21:564-579 (2002), Hahn et al, Nature 400:464-468 (1999), Shay and Bacchetti, Eur. J. Cancer 33:787-791 (1997)), making constitutive expression of this potentially dangerous. Transient expression of hTERT has been argued as a means to overcome this limitation but the period of time that hTERT is expressed may not be sufficient to elongate telomeres enough for clinical applications or long term sustainability in the human body. However, transient expression of a nucleic acid sequence of the invention, and production of the encoded chimeric molecule, can be used for such applications.

Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows.

## EXAMPLE

### Experimental Details

Constructs (Fig. 1)

A chimeric protein of hPot1 (accession number NM_015450) was engineered to contain a FLAG-epitope tag fused to the N-terminus of hTERT (accession number AF018167) previously engineered to contain a FLAG-epitope tag (Armbruster et al, Mol. Cell Biol. 21:7775-7786 (2001)). Specifically, hPot1 cDNA in

the plasmid pQE30-hPot1 (gift of Dr. Tom Cech, Boulder CO) was PCR amplified with primers 5'-cgGGATCCTACGTAGCTAGCatgGACTACAAAGACGATGACGACAAGTCTTT GGTTCCAGCAACAAAT (SEQ ID NO:2) and 5'-cgacGTCGACtaaaTACGTAcGATTACATCTTCTGCAACTGTG (SEQ ID NO:3) to generate an N-terminal flag epitope tagged hPot1 lacking a terminating codon (flag-hPot1). The resultant product was subcloned into the *Bam*HI/*Sal*I sites of pBluescript SK- (Stratagene) and sequenced to verify that the sequence was correct. Flag-hPot1 was then excised from this plasmid with *Sna*BI and cloned into the same site in the retroviral construct pBabepuro-flag-hTERT (Armbruster et al, Mol. Cell Biol. 23:3237-3246 (2003)), generating pBabepuro-flag-hPot1-flag-hTERT. Similarly, the same Flag-hPot1 fragment was cloned into the *Sna*BI site of pBabepuro-flag-hTERT$_{+128}$ (Armbruster et al, Mol. Cell Biol. 23:3237-3246 (2003)), generating the plasmid pBabepuro-flag-hPot1-flag-hTERT$_{+128}$. Plasmids pBabepuro, pBabepuro-flag-hTERT$_{+128}$, and pBabepuro-flag-hTERT were previously described (Armbruster et al, Mol. Cell Biol. 23:3237-3246 (2003), Morgenstern and Land, Nucleic Acids Res. 18:1068 (1990)).

Cell culture and immortalization (Figs. 2 and 4)

At late passage, cells of the SV40 early region transformed human embryonic kidney cell line HA5 (Stewart and Bacchetti, Virology 180:49-57 (1991)) were infected with amphotropic retrovirus derived from the constructs pBabepuro-flag-hPot1-flag-hTERT and pBabepuro-flag-hPot1-flag-hTERT$_{+128}$ or, as

controls, pBabepuro, pBabepuro-flag-hTERT$_{+128}$, and pBabepuro-flag-hTERT using methods identical to those previously described (Armbruster et al, Mol. Cell Biol. 21:7775-7786 (2001)). Stably infected polyclonal populations were selected in media supplemented with 1.0 µg/ml puromycin (Sigma). Population doubling (PD) 0 was arbitrarily assigned to the first confluent plate under selection. The resultant five cell lines were continually passaged 1:8 under selection until either crisis or until the culture divided 2.5 times more than vector-control cell lines. Crisis was defined as the period when cultures failed to become confluent within 4 weeks and displayed massive cell death.

Protein expression (Fig. 2)

150 µg of soluble lysate from the aforementioned five cell lines was separated by SDS-PAGE, and immunoblotted as previously described (Armbruster et al, Mol. Cell Biol. 23:3237-3246 (2003)) with the primary mouse monoclonal antibodies anti-FLAG M2 (Sigma) and anti-actin C-2 (Santa Cruz Biotechnology Inc.) to detect flag-hTERT or actin, respectively, followed by incubation with the goat anti-mouse IgG-HRP (81-6520) (Zymed Laboratories Inc.). Proteins were then detected with ECL reagent following the manufacturer's protocol (Amersham Pharmacia Biotech).

Detection of *in vitro* telomerase activity (Fig. 2)

0.2 μg of lysates prepared from the aforementioned five cell lines at early passage were assayed for telomerase activity using the telomeric repeat amplification protocol (Kim and Wu, Nucleic Acids Res. 25:2595-2597 (1997)). Reaction products were resolved on 10% polyacrylamide gels, dried and exposed to a phosphorimager screen to visualize enzyme activity, as previously described (Kim and Wu, Nucleic Acids Res. 25:2595-2597 (1997)).

Telomere length analysis (Fig. 3)

5 μg of genomic DNA isolated from late passage HA5 cells stably expressing flag-hTERT, flag-hPot1-flag-hTERT or flag-hPot1-flag-$hTERT_{+128}$, was digested with *Hinf*I and *Rsa*I restriction enzymes to release terminal restriction fragments containing telomeric DNA, Southern hybridized with a $^{32}P$-labeled telomeric $(C_3TA_2)_3$ oligonucleotide and exposed to a to phosphorimager screen (Molecular Dynamics), similar to methods previously described (Counter et al, EMBO J. 11:1921-1929 (1992)).

Results

Fusion of hPot1 to hTERT.

hPot1 has been shown to co-localize with the telomeric binding protein TRF2 by immunoflourescence analysis *in vivo* (Baumann et al, Mol. Ce.. Biol. 22:8079-8087 (2002)), and associate with the G-rich

strand of telomeric DNA *in vitro* (Baumann and Cech, Science 292:1171-1175 (2001)). Moreover, it has been shown that hPot1 co-immunoprecipitates specifically with telomeric DNA upon chemical crosslinking, indicating that hPot1 is a *bona fide* telomere binding protein. Because hPot1 binds single stranded telomeric DNA (Baumann and Cech, Science 292:1171-1175 (2001)) and because the budding yeast orthologue Cdc13p is known to cap the ends of the G-strand of telomeres (Nugent et al, Science 274:249-252 (1996)), the question prsented was whether a fusion of hPot1 to the hTERT catalytic subunit of human telomerase (Meyerson et al, Cell 90:785-795 (1997)) might direct telomerase more often to telomeres, thereby greatly elongating telomeric DNA. To this end, a novel cDNA was created that encoded FLAG-epitope tagged hPot1 fused in frame FLAG-epitope tagged hTERT, termed hPot1-hTERT.

hPot1-hTERT is expressed in human cells and retains telomerase catalytic activity.

To ascertain if, as predicted, hTERT targeted to telomeres by hPot1 results in telomere elongation, the cell strain HA5 was stably infected with retroviruses derived from constructs encoding the fusion protein or, as controls, hTERT or an empty vector. HA5 are a human embryonic kidney cell strain transformed with the early region of SV40 (Stewart and Bacchetti, Virology 180:49-57 (1991)). Since these cells lack telomerase activity, they

lose telomeric DNA each cell division until a critically short length is reached, which leads to genomic instability and cell death (Counter et al, EMBO J. 11:1921-1929 (1992)) and hence can be used to monitor the ability of telomerase to function *in vivo*.

A protein of the predicted combined molecular weight of hTERT and hPot1 is specifically detected by immunoblotting with an antibody directed against the FLAG epitope engineered in the hPot1-hTERT fusion protein in cells infected with the retrovirus encoding hPot1-hTERT (Fig. 2A), indicating that the fusion protein is indeed expressed. As controls, immunoblot analysis confirmed the expression of hTERT or no transgene in the two control cultures (Fig. 2A).

Ectopic expression of hTERT is known to restore telomerase activity in HA5 cells, which normally lack hTERT (but express the hTR RNA subunit) (Meyerson et al, Cell 90:785-795 (1997)). Indeed, extracts isolated from both hTERT and hPot1-hTERT expressing cells, but not vector control cells, supported the elongation of a telomeric primer *in vitro* by the addition of telomeric repeats, which when PCR-amplified and resolved form a ladder indicative of telomerase catalytic activity (Fig. 2B). Thus, fusion of hPot1 to hTERT did not disrupt the catalytic activity of hTERT, arguing that the fusion protein is functional.

hPot1 recruitment of hTERT to telomeres causes dramatic telomere elongation.

Having ascertained that HA5 cells express the hPot1-hTERT fusion protein, the question presented was whether hTERT was better able to elongate telomeres when fused to hPot1. DNA was therefore isolated from late passage HA5 cells expressing hPot1-hTERT, or as a control hTERT, and digested with restriction enzyme *Hin*F1 and *Rsa*I to liberate telomere containing fragments, which when resolved on agarose gels were detected by Southern hybridization with a telomere specific probe. It was found, as previously reported (Counter et al, Proc. Natl. Acad. Sci. USA 95:14723-14728 (1998)), that HA5 cells expressing hTERT stabilized telomeres at a short length (Fig. 3). On the other hand, the same cell expressing the fusion protein exhibited greatly elongated telomeres, reaching telomere lengths at least three times larger than hTERT control cells (Fig. 3). Thus, the hPot1-hTERT fusion protein is far superior at elongating telomeres compared to the wild-type hTERT protein.

The hPot1-hTERT fusion protein immortalizes human cells.

To determine if the fusion protein had any adverse affects on cell immortalization, the lifespan was monitored of HA5 cells expressing hPot1-hTERT or as controls, hTERT or no protein (vector). It was found, as expected, that vector control HA5 cells were mortal (Fig. 4), owing to the

fact that telomere shortening was not arrested (Counter et al., EMBO J. 11:1921-1929 (1992)). On the other hand, cells expressing hTERT continued to proliferate well beyond the point when vector control cells perished at crisis (Fig. 4) due to the arrest of telomere shortening (Fig. 3). Importantly, cells expressing hPot1-hTERT grew similarly to hTERT-expressing cells, indicating that the fusion protein was not lethal and that it generated functional (but long) telomeres (Fig. 4). hPot1 fusion rescues the inability of a telomere-targeting mutant of hTERT to elongate telomeres.

A mutant of hTERT ($hTERT_{+128}$) was recently described with intact catalytic activity *in vitro* but defective in telomere elongation (Armbruster et al, Mol. Cell. Biol. 21:7775-7786 (2001)). Targeting this crippled enzyme to telomeres by fusion with the telomeric protein TRF2, which binds close but presumably not at the very ends of telomeres, partially rescued this defect. Cells expressing this fusion protein were immortal, but exhibited very short telomeres and a decreased proliferative rate (Armbruster et al, Mol. Cell. Biol. 23:3237-3246 (2003)). It was reasoned that since hPot1 may bind very close, if not to the ends of telomeres, fusion of hPot1 to $hTERT_{+128}$ may completely rescue this mutation. Therefore, hPot1-$hTERT_{+128}$ and as a control $hTERT_{+128}$ retroviral expression constructs were created from which retroviruses were generated. HA5 cells were then stably infected with these viruses and confirmed by

immunoblot analysis to express either the $hTERT_{+128}$ or the hPot1-$hTERT_{+128}$ proteins (Fig. 2A). Extracts isolated from these two cell lines also showed equal levels of telomerase activity, indicating that the fusion did not disrupt the catalytic activity of $hTERT_{+128}$.

Control cultures grew as expected, with $hTERT_{+128}$-expressing cells entering crisis and dying with short telomeres (Fig. 4). However, cells expressing hPot1-$hTERT_{+128}$ proliferated indefinitely (Fig. 4) and exhibited a dramatic increase in telomere length (Figure 3). Thus, targeting $hTERT_{+128}$ to telomeres by hPot1 not only rescued the inability of the crippled telomerase to replicate telomeres, but also greatly enhanced the ability of this hTERT protein to elongate this DNA. The data indicate that the hPot1-hTERT generates a remarkably active enzyme capable of elongating telomeres even when hTERT is critically mutated.

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All documents cited above are hereby incorporated in their entirety by reference.